ChemBioChem **2004**

Supporting Information

for

New Rapamycin Derivatives by Precursor-Directed Biosynthesis

Philip A.S. Lowden*, Günter A. Böhm, Su Metcalfe, James Staunton, Peter F. Leadlay*

Maintenance of Bacterial Strains

Rapamycin and its derivatives were produced by *Streptomyces hygroscopicus* (ATCC 29253), or by two mutant strains provided by the Institute for Drug Research (IDR) in Budapest – GYKI-456UV and GYKI-84UV-NTG129. No significant differences in rapamycin production were observed under the growth conditions used. *Streptomyces hygroscopicus* cultures were maintained on SY agar plates at room temperature. New plates were spread when spores developed and the surface of the plates started to change from white to grey (1-2 weeks).

Growth of *S. hygroscopicus* in Shake Flasks

Liquid seed cultures (RAP seed medium) were inoculated with spores (~20 cm^2 plate per litre of culture) and grown in baffled flasks (100 ml culture in 500 ml flasks, 500 ml culture in 2 l flasks) at 240 rpm and 28°C for 1-2 days. TSB production medium was inoculated with 5% v/v seed medium and grown in baffled flasks (100 ml culture in 500 ml flasks, 500 ml culture in 2 l

flasks) at 240 rpm at 26°C until the pH had risen to between 6 and 8 (4-5 days).

SY Agar

Soluble starch	1%
Yeast extract (DIFCO)	0.1%
K_2HPO_4	0.1%
$MgSO_4.7H_2O$	0.1%
NaCl	0.3%
TES buffer	30 mM
Agar (Bacto)	2.2%

Adjusted to pH 7.4 before sterilisation.

RAP Seed Medium

Casein Hydrolysate (DIFCO or OXOID)	1%
Yeast Extract (DIFCO or OXOID)	0.1%
Peptone (DIFCO or OXOID)	0.1%
$MgSO_4.7H_2O$	0.01%
K_2HPO_4	0.2%
Glucose (sterilised separately as 50% solution)	2%

Adjusted to pH 7.2 with conc. sulphuric acid before sterilisation.

TSB Production Medium

Tryptic Soy Broth (DIFCO)	3%
$FeSO_4.7H_2O$	0.01%
Glucose (sterilised separately as 50% solution)	1.5%

Adjusted to pH 6.0 with conc. sulphuric acid before sterilisation.

Feeding of Precursors

Precursors were added 24 hrs after inoculation in concentrated ethanol solution through a Sartorius Minisart 0.2 micron filter to give a concentration of 1 mM in the culture medium. For

small scale (100 ml culture) feeds of ethanol solutions, precursors were added by pipette using sterile pipette tips.

General Procedure for Isolation of Metabolites

Fermentation broth from small scale growths was centrifuged at 8000 rpm for 15 mins, the micelial pellet retained, washed with the same volume of distilled water and centrifuged again. The pellet was then extracted twice with methanol (1 ml per 2 ml culture) for 1 hr at room temperature or overnight at 4 °C. Analytical HPLC or LCMS was performed at this stage. The methanolic extract was filtered and concentrated under reduced pressure until only the residual water remained. This was then resuspended in water (1 ml per 5 ml methanol extract) and extracted three times with an equal volume of ethyl acetate. The ethyl acetate extract was dried over sodium sulphate, filtered and the solvent removed under reduced pressure under reduced pressure. Extracts were cleaned of polar components by flash silica column chromatography using 1:1 acetone:hexane, collecting fractions with compounds of R_f between 0.2 and 0.4. Purification was completed by preparative reverse-phase HPLC.

Analytical HPLC Methods

Analytical HPLC was carried out on a Hewlett Packard HP1090 instrument, with diode array detection. Retention times were determined by following the absorption at 277 nm. LCMS data were obtained on a Finnigan-MAT LCQ instrument linked to a Hewlett Packard HP1050 HPLC system.

Column: Applied Biosystems Brownlee Column (SPHERI-5, RP-18, 5 µ, 100×4.6 mm)

Time / mins	MeOH/%	H_2O/%
0	75	25
14	100	0

Preparative HPLC Methods

Preparative HPLC was carried out on a Gilson modular system with UV detection at 277 nm.

Column: HPLC Technology Spherisorb (5 μ, ODS, 250×20 mm).

Method 1:

Time / mins	MeOH/%	H_2O/%
0	80	20
15	100	0
18	100	0

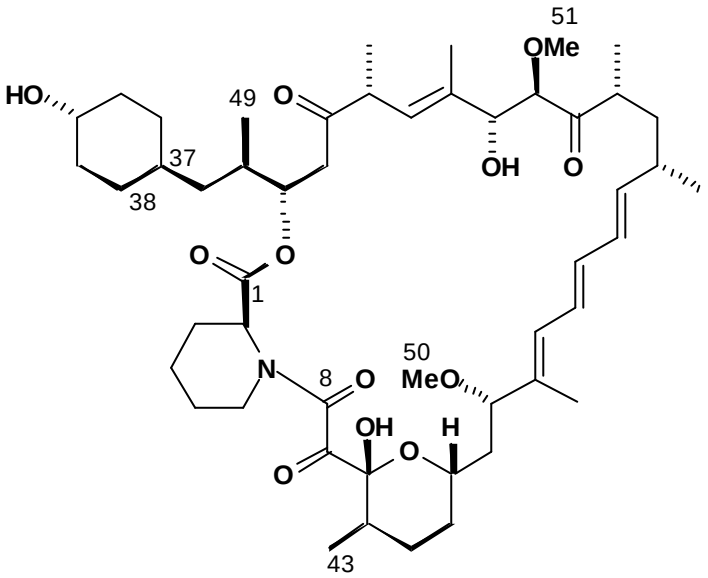
Method 2:

Isocratic elution: 75% MeOH, 25% H_2O.

Method 3:

Isocratic elution: 65% MeCN, 35% H_2O.

Cyclohexanecarboxylic Acid Feed – Compound 3



Analytical HPLC RT = 8.02 mins. (rapamycin at 7.85 mins. in same HPLC sequence). Purified using preparative method 1.

$[\alpha]_D$ = -51.1° (c = 0.89, $CHCl_3$)

UV λ_{max} (MeOH)/nm: 268, 278, 288.

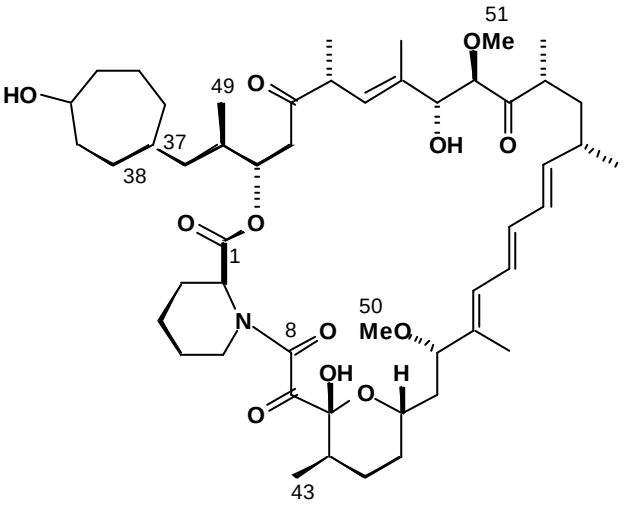
IR ν_{max} ($CHCl_3$)/cm^{-1}: 3436w (OH), 2931s (CH), 1721s (C=O), 1624s, 1452s, 1375m, 1328w.

	^{13}C δ/ppm	^{1}H δ/ppm	Multiplicity	J/Hz
1	169.3			
2	51.3	5.28	d	5
3	27.1	2.35, 1.76	m	
4	20.6	1.78, 1.47	m	
5	25.3	1.75, 1.46	m	
6	44.2	3.57, 3.45	d, d	16, 16
8	166.8			
9				
10	98.1			
11	33.8	1.97	m	
12	27.3	1.61, 1.57	m	
13	31.2	1.59, 1.31	m	
14	67.2	3.84	m	
15	38.9	1.83, 1.52	m	
16	84.4	3.66	t	8
17	135.2			
18	129.6	5.96	d	10
19	126.5	6.38	dd	15 10
20	133.6	6.31	dd	15 10
21	130.2	6.14	dd	15 10
22	140.1	5.55	dd	15 9
23	35.2	2.30	m	
24	40.2	1.48, 1.19	m	
25	41.5	2.76	m	
26	215.6			
27	84.9	3.70	d	6
28	77.1	4.15	d	6
29	136.0			
30	126.7	5.40	d	10
31	46.5	3.32	m	
32	208.4			
33	40.8	2.73, 2.60	dd, dd	17 6, 17 6
34	75.9	5.16	q	5
35	33.1	1.95	m	
36	38.5	1.19, 1.05	m	
37	34.0	1.96		
38	30.6	1.75, 0.82	m	

39	35.5	1.95	m	
40	70.9	3.53	m	
41	35.5	1.95		
42	32.4	1.72, 0.99		
43	16.2	0.95	d	7
44	10.2	1.65	s	
45	21.5	1.04	d	7
46	13.7	0.99	d	7
47	13.2	1.74	s	
48	16.0	1.10	d	7
49	15.8	0.90	d	7
50	55.9	3.13	s	
51	59.3	3.33	s	

MS (ES) m/z$^-$: 882.4 (M-H), 864.2 (M-H_2O), 753.1 (M-130 (pipecolate)), 590.1 (C-1 to C-27), 291.1 (C-28 to C-42).
(Found (FAB): M+Na^+ = 906.53030, $C_{50}H_{77}NNaO_{12}$ requires m/z = 906.53436).

Cycloheptanecarboxylic Acid Feed – Compound 4



Analytical HPLC RT = 9.00 mins. (rapamycin at 7.85 mins. in same HPLC sequence). purified using preparative method 2.
UV λ_{max} (MeOH)/nm: 268, 278, 288.
IR ν_{max} ($CHCl_3$)/cm^{-1}: 3436w (OH), 2931s (CH), 1721s (C=O), 1624s, 1452s, 1375m, 1328w.

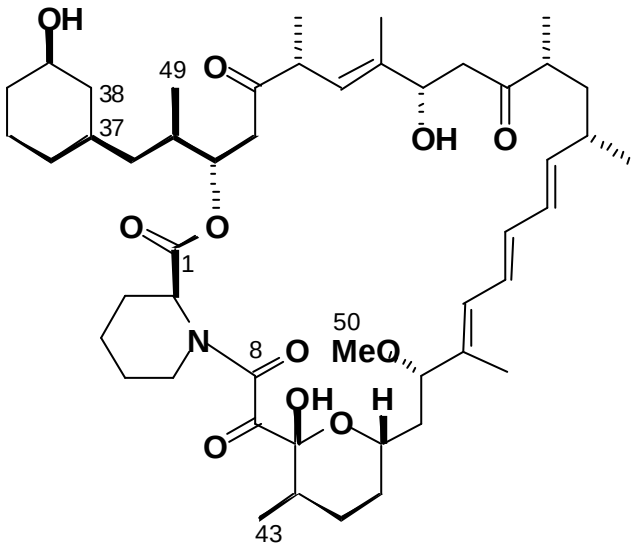
	^{13}C δ/ppm	^{1}H δ/ppm	Multiplicity	J/Hz
1	169.3			
2	51.0	5.28	d	15
3	27.0	2.34, 1.59	m	
4	20.3	1.76, 1.49	m	
5	25.1	1.75, 1.47	m	
6	44.0	3.58, 3.43	d, d	11, 11
8	171.5			
9	*			
10	98.4			
11	33.3	1.96	m	
12	26.9	1.60, 1.59	m	
13	31.1	1.60, 1.32	m	
14	67.1	3.85	m	
15	38.0	1.84, 1.53	m	
16	84.4	3.64	t	7
17	135.3			
18	129.5	5.96	d	11
19	126.2	6.38	dd	15 11
20	133.6	6.30	dd	15 10
21	129.9	6.14	dd	15 10
22	140.3	5.54	dd	15 9
23	35.0	2.30	m	
24	40.2	1.50, 1.20	m	
25	40.9	2.72	m	
26	215.8			
27	84.6	3.70	d	6
28	77.3	4.15	d	6
29	135.8			
30	126.6	5.40	d	10
31	46.5	3.32	m	
32	208.0			
33	40.8	2.75, 2.57	dd, dd	17 6, 17 6
34	75.7	5.17	q	5
35	33.0	1.94		
36	39.3	1.52, 0.99		
37	*	*		
38/42	35.5	1.74, 1.16		

39/41	37.4	1.84, 1.59		
40	72.8	3.80		
41/39	35.7	1.95, 1.43		
42/38	28.0	1.65, 0.94		
43	*	*		
44	16.0	0.95	d	7
45	9.9	1.65	s	
46	21.4	1.05	d	7
47	13.7	0.99	d	7
48	12.7	1.74	s	
49	15.8	1.10	d	7
50	15.6	0.89	d	7
51	55.6	3.13	s	
52	59.3	3.33	s	

*- could not be unambiguously assigned

MS (ES) m/z$^-$: 896.5 (M-H), 878.2 (M-H_2O), 767.1 (M-130 (pipecolate)), 590.1 (C-1 to C-27), 305.1 (C-28 to C-42). (Found (FAB): M+Na^+ = 920.54840, $C_{51}H_{79}NNaO_{12}$ requires m/z = 920.55001).

Cyclohex-1-enecarboxylic Acid Feed – Compound 5



Analytical HPLC RT = 8.34 mins. (rapamycin at 7.14 mins. in same HPLC sequence). Purified using preparative method 3.

$[\alpha]_D$ = -67.1° (c = 0.62, $CHCl_3$).

UV λ_{max} (MeOH)/nm: 268, 278, 288.

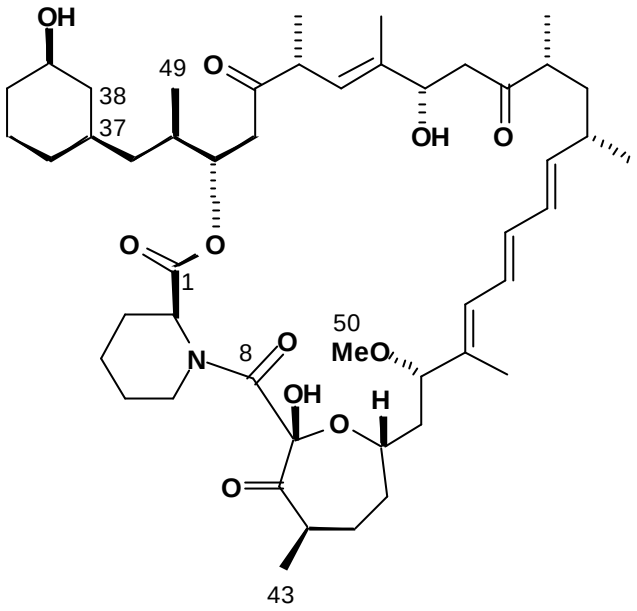
IR ν_{max} ($CHCl_3$)/cm^{-1}: 3516w (OH), 2931s (CH), 2863m (C=O), 1719s (C=O), 1643s, 1452s, 1377m, .

	^{13}C δ/ppm	^{1}H δ/ppm	Multiplicity	J/Hz
1	169.2			
2	51.4	5.21	m	
3	26.4	2.31, 1.74	m	
4	20.4	1.76, 1.47	m	
5	25.0	1.64, 1.48	m	
6	44.0	3.53, 3.22	m	
8	166.4			
9	194.7			
10	98.3			
11	34.2	2.05	m	
12	26.9	1.62, 1.62	m	
13	30.8	1.71, 1.29	m	
14	67.4	4.00	m	
15	39.5	1.89, 1.42	m	
16	83.6	3.59	m	
17	136.2			
18	128.9	5.96	d	11
19	126.6	6.35	dd	15 11
20	133.1	6.21	dd	15 11
21	129.6	6.07	dd	15 11
22	140.6	5.38	m	
23	37.5	2.18	m	
24	40.3	1.74, 1.32	m	
25	45.6	2.50	m	
26	215.8			
27	47.2	2.64, 2.53	m	
28	71.7	4.34	m	

29	139.1			
30	123.9	5.39	d	9
31	45.9	3.22	m	
32	207.8			
33	41.0	2.67, 2.55	m	
34	74.8	5.23	m	
35	33.1	1.92	m	
36	39.2	1.25, 1.03	m	
37	33.4	1.95	m	
38	43.4	1.95, 0.88	m	
39	70.3	3.56	m	
40	35.4	1.96, 1.12	m	
41	23.8	1.76, 1.23	m	
42	31.2	1.64, 0.69	m	
43	15.9	0.93	d	7
44	10.1	1.65	s	
45	21.6	1.02	d	7
46	17.2	1.04	d	7
47	12.2	1.56	s	
48	16.1	1.11	d	7
49	15.3	0.88	d	7
50	55.7	3.11	s	

MS (ES) m/z^-: 852.2 (M-H), 834.2 (M-H_2O), 723 (M-130 (pipecolate)), 560 (C-1 to C-27), 291 (C28 to C-42).
(Found (FAB): $M+Na^+$ = 876.52980, $C_{49}H_{75}NNaO_{11}$ requires m/z = 876.52380).

Cyclohex-1-enecarboxylic Acid Feed – Compound 6



Analytical HPLC RT = 9.08 mins. (rapamycin at 7.85 mins. in same HPLC sequence). Purified using preparative method 3.

$[\alpha]_D$ = -87.5° (c = 0.36, $CHCl_3$).

UV λ_{max} (MeOH)/nm: 268, 278, 288.

IR ν_{max} ($CHCl_3$)/cm^{-1}: 3436m br (OH), 2932s (CH), 2860m (CH), 1721s (C=O), 1632s, 1454m, 1377m, 1282m.

	^{13}C δ/ppm	^{1}H δ/ppm	Multiplicity	J/Hz
1	169.6			
2	51.6	5.12	br d	5.5
3	26.2	2.32, 1.53	br d, m	13
4	20.5	1.68, 1.30	m	
5	24.9	1.57, 1.50	m	
6	43.2	4.54, 2.70	br d, m	14
8	167.7			
9	97.5			
10	210.3			
11	43.1	3.24	m	
12	34.6	1.84, 1.33	m	
13	32.3	1.90, 1.39	m	

14	72.9	3.70	td	10 4
15	41.9	2.09, 1.26	m	
16	83.4	3.53	m	
17	138.2			
18	127.5	5.96	d	11
19	126.9	6.29	dd	14 11
20	132.4	6.08	dd	14 11
21	129.1	6.03	m	
22	141.5	5.24	dd	14 10
23	40.1	2.02	m	
24	40.2	2.02, 1.36	m	
25	46.4	2.43	m	
26	215.7			
27	48.6	2.64, 2.52	m	
28	69.7	4.33	m	
29	139.1			
30	122.0	5.37	d	10
31	47.2	3.10	m	
32	208.1			
33	40.3	2.60, 2.48	m	
34	73.7	5.50	dt	11 3
35	32.8	1.92	m	
36	39.5	1.16, 1.01	m	
37	33.3	1.91	m	
38	43.0	1.97, 0.86	m	
39	70.2	3.56	m	
40	35.4	1.96, 1.12	m	
41	23.8	1.74, 1.23	m	
42	31.5	1.62, 0.68	m, qd	13 3
43	16.5	1.24	d	6
44	10.2	1.69	s	
45	21.5	1.01	m	
46	19.1	1.03	m	
47	14.1	1.50	s	
48	15.0	1.02	m	
49	14.7	0.85	d	6
50	56.1	3.08	s	

MS (ES) m/z^-: 852.2 (M-H), 834.2 (M-H_2O), 723 (M-130 (pipecolate)), 560 (C-1 to C-27), 291 (C28 to C-42).
(Found (FAB): $M+Na^+$ = 876.52980, $C_{49}H_{75}NNaO_{11}$ requires m/z = 876.52380).